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Attn. Mr. Mossier, B

12 May 2005

Fax no: 00 49 8923994465

**Re: Reply to second drawn written opinion – WO – issued under Rule 66.4 PCT  
International application no. PCT/ES2004/070001**

Dear Sirs,

We reply in due time to the 2<sup>nd</sup> WO issued by the EPO acting as IPEA in the framework of the PCT. After having maintained an informal communication by phone, under Rule 66.6 PCT, with the examiner in charge of the IPE of present IA, Ms. Mossier, we wish to thank her for her understanding and comprehension by giving us an additional opportunity to submit further arguments and amendments under Rule 66.4 PCT, in defense of the patentability of the IA as now claimed in the newly amended set of claims.

We notice the acknowledgement of novelty and inventive step made by the examiner in her 1<sup>st</sup> WO, concerning claims 7-14 (see paragraph V3.2, last sentence). Based on that we have further amended the previously submitted set of claims by trying to overcome the still outstanding objections raised by the examiner.

#### Support for the amendments further introduced

+ We have replaced the term "Assay kit" in claims 1-6 and 12 by the term "Microarray". Support for that amendment may be found in the specification as formerly filed (see pg. 14, lines 6-10, 14 and pg. 15 line 9 to pg. 16 line 5, pg. 16 line 23, pg. 17. line 22, pg. 18, line 12, .... pg. 61, lines 10-14 for the explanatory legend foot-note of Fig. 3). The term "microarray" is also mentioned all along the specification as "DNA-array", "bio-chip", etc....All the terms used are synonyms defining an assay kit consisting in a large

amount of probes (oligonucleotides) able to hybridize with a plurality of mutations existing in the LDL-r gene sequence, which determine the in vitro diagnostic of FH, therefore being able to detect specifically any of those mutations.

+ We have also replaced the term “hybridizing” in claims 1, 3, 4 and 13 by the term “able of specifically detecting”. Support for that amendment may be found in the specification as formerly filed (see pg. 14, lines 6-10 and pg. 15 lines 21-33) wherein each 2 pairs of oligonucleotides are designed for specific hybridization with each mutation.

#### Novelty (Art. 33.1 and 33.2 PCT)

##### Newly amended claim 1 (and dependent claims 2-6 thereof)

The microarray device of the invention allows the multiple detection of several mutations or Single Nucleotide Polymorphisms (SNPs) in a single sample, with a single assay. That means that, by the single device of the invention claimed, in a single step, a DNA extracted from a blood sample of a patient suspect of FH, can be assayed for a multiplicity of new, undisclosed mutations, all of them located in the same gene sequence (SEQ ID NO: 1, the DNA sequence of LDL-r human gene) and whose occurrence in the patient's DNA sample, individually, implies the diagnosis of the same sickness: familial hypercholesterolemia (FH). It is true that either D1 or D2 disclose other different LDL-r gene mutations which may also be related with developing or suffering FH, but none of those prior art documents discloses a microarray capable of detecting a multiplicity of mutations. Those prior art documents disclose instead assay devices for detecting, one by one, in each sample, a single mutation. That implies to carry out repetitive analysis for the same patient's sample for each mutation. D1 and D2 both show assays for punctual mutation analysis based in Denaturing Gradient Gel Electrophoresis (DGGE). Those kind of assays techniques are expensive, time-consuming and they have a more limited number of samples being processed at the same time, as compared with the microarray of the invention. DGGE is only indicated for screening populations seeking the identification of mutations, but not for large scale processing of FH patient samples for in vitro diagnosis.

Moreover, by removing the term “hibridizing” from the amended claims when defining the oligonucleotides used for each mutation's detection, we have tried to overcome the novelty objection raised by the examiner in the sense that, without hybridization conditions, the oligonucleotides used in the invention might also hybridize with mutations already disclosed in the prior art. Now the concept of hybridization has been replaced by the technical concept of ability of specifically detecting a given mutation. That replacement is of not superfluous nature in view of the interpretation given in the specification as formerly filed in pg. 15, lines 21-25, about what is meant by a specific detection of a given mutation in LDL-r gene (SEQ ID NO: 1). The oligonucleotides used have been designed to specifically detect the target mutation in the central position of its oligonucleotide sequence, hence avoiding hybridization with adjacent bases, even if those adjacent bases were also mutated as the case 4 of D1 discussed in the 2<sup>nd</sup> WO.

### Inventive step (Art. 33.1 and 33.3 PCT)

Newly amended claim 1 (and dependent claims 2-6 thereof)

The technical problem solved by the invention is to provide an alternative genetic assay for diagnosis of FH by testing in a single DNA sample, through a single process, a multiplicity of new mutations of LDL-r gene, proven to be linked to the development of that sickness. For that purpose, Spanish FH patients have been used as source of new mutations and the corresponding probes or oligonucleotides have been developed for specifically detecting the aforesaid new mutations. However, although the new mutations have been identified in the Spanish population, that means that the probability of occurrence of those new mutations is higher into Spanish FH patients than in other populations world-wide. However, by using the microarray of the invention, the probabilities of diagnosis of FH are increased simply because the number of mutations on which the assay is based has been enlarged. It should bear in mind that, dependent claims 3 and 4, encompass, together with the new mutations detected substantially in the Spanish population, other mutations linked to FH and identified in human populations world-wide, other than Spanish.

D1 or D2, or combinations thereof, neither disclose nor suggest a microarray based in a technical approach as the one now claimed for the present invention. They are only intended for screening of Japanese or Finish populations, respectively, in the search of new mutations in LDL-r gene, of high national prevalence on their respective populations. But they are far away of even suggesting or persuading to the man skilled in the art to seek for a microarray capable of simultaneous detection of a plurality of mutations in the LDL-r gene, based in the Spanish population.

### Unity of invention (Rules 13.1. and 13.2. PCT)

Once proven novelty and inventive step of newly amended claim 1, the lack of unity problem raised "a posteriori", lacks legal grounds in our opinion. Concerning the unity between amended independent claims 1, 7, 9 and 13, we refer to the statement made in pg. 8, lines 27-30 of the specification as formerly filed. We are claiming a single underlying inventive concept comprising: an in vitro method of diagnosis of FH based in the detection of new and inventive mutations existing in the DNA sequence of the LDL-r gene, a microarray as preferred physical embodiment for carrying out the aforesaid method in vitro, the use of oligonucleotides for specifically detecting the above mentioned mutations and the specific oligonucleotides employed in the claimed microarray.

### Clarity and Sufficiency of Disclosure (Art. 5 and 6 PCT)

We have added a reference to the SEQ ID NO:1, besides any mention existing in the amended claims to the LDL-r gene, to better identify the positions of the different mutations, as suggested by the examiner.

We have removed the term "hybridizing" referred to the oligonucleotides used in the method and/or the microarray. We wish to point out that the hybridizing conditions are

disclosed in the specification as formerly filed (see pg. 14 line 6 to pg. 16, line 5 and Fig. 3). Any other data needed by the man skilled in the art to put into practice the invention, with regard to the probes/oligonucleotides hybridization with the DNA mutated sequences present in the patient samples assayed in the microarray, is given by each supplier of the hybridization stations and they are not subject of invention but routine information (assumed as common knowledge) to be provided to the technicians operating those automatic hybridization stations when they are purchased, as a sort of instructions' manual. Nevertheless, to avoid any ambiguity concerning that term, we have replaced it.

Hopefully, with the newly amended set of claims, any outstanding objection would be overcome and the IPER to be issued by the EPO would recognize the novelty and inventive step of the IA, in its entire set of claims as finally amended.

Thanking you so much, once more time, for your efforts to have been able of giving us an additional opportunity to submit further amendments and arguments, we remain,

Very truly yours,

ELZABURU

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Dr. Manuel Illescas

ANNEX:

- Newly amended set of claims (replacement pages 63-67)

## AMENDED CLAIMS

- 1.- Microarray characterized by comprising oligonucleotides able of specifically  
 5 detecting in the DNA sequence of LDL-r gene (SEQ ID NO: 1) any of the  
 mutations selected from: (-23)A>C, 1054 del11, 108delC, 1197de19, 1207de1T,  
 1432delG, 191-2delAinsCT, 2184delG, 231delC, 2399del5ins4, 313+1insT,  
 338del16, 509insC, 675del15, 684dup12, 941-39>T, C195R, C255G, C319Y,  
 D157G, D630N, E291X, H635N, N59K, T41M, W515X, Y379X, Y421X,  
 10 T433N, 818de18, 1423delGC/insA, 1204insT, 451de13, G516X, 2389+4A>G,  
 1815del11, 1186+5G>A, T740M, I771T, R279G, T446I, H562Q, C74Y, D686Y,  
 G(-2)R, E579D, S205C, D200V, V766E, L(-6)P, 2544insC, C42Y, 2389+3A>C,  
 [1587-5de15;1587del31].
- 15 2.- Microarray according to claim 1 characterized by comprising at least an  
 oligonucleotide selected from: SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:16,  
 SEQ ID NO:17, SEQ ID NO:24, SEQ ID NO:29, or at least one from SEQ ID  
 NO:37 to SEQ ID NO:147 or from SEQ ID NO:154 to SEQ ID NO:259.
- 20 3.- Microarray according to any of the claims 1 or 2 characterized by further  
 comprising oligonucleotides able of specifically detecting in the DNA sequence  
 of LDL-r gene (SEQ ID NO: 1) any of the mutations selected from: 2393del19, (-  
 42)C>G, (-49)C>T, 1045delC, 1061-8 T>C, A378T, C358R, 1358+1G>A, 1706-  
 10G>A, 1845+1G>C, 2085del19, 211delG, 2140+5G>A, 2207insT, 2390-1G>C,  
 25 313+1G>C, 313+1G>A, 518delG, 7delC, 872delC, 884delT, 920ins4, A519T,  
 C113W, C255X, C281Y, C297F, C347Y, C371X, C646Y, C677Y, C68W,  
 C74G, C95R, D151N, D200G, D200Y, D280G, E10X, E246A, E256K, F634L,  
 G322S, G352D, G571E, N543H, N804K, Q12X, Q133X, Q357P, Q427X, Q71E,  
 R395Q, R574W, R612C, S156L, S205P, T413K, T7051, V502M, W(-18)X,  
 30 W541X, D679E, 1359-1G>A, C127R, 681ins21, C122X, V408M, G528D,  
 D412H, N619N, E80K, L534P, L621S, C356Y, R329X, G248D, C201Y,

313+5G>A, C358Y, C331R, D157N, V776M, P664L, W462X, Q328X, L584P, R395W, G314V, W469X, P678L, R612H, R236W.

4.- Microarray according to any of the claims 1 to 3 characterized by further  
5 comprising oligonucleotides able of specifically detecting in the DNA sequence  
of LDL-r gene (SEQ ID NO: 1) any of the polymorphisms selected from: 81T>C  
BstUI Exon 2, 1060+10G>C SmaI Exon 7, 1171G>A Stul Exon 8, 1413G>A  
Ddel Exon 10, 1617C>T BstNI Exon 11, 1725C>T SSCP Exon 12, 1771C>T  
HincII Exon 12, 1959 T>C AvaII Exon 13, 2232G>A MspI Exon 15.

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5.- Microarray according to any of the claims 1 to 4 characterized by comprising  
at least an oligonucleotide selected from: SEQ ID NO: 2, SEQ ID NO:3, SEQ ID  
NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO: 9, SEQ ID  
NO:10, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ  
15 ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22,  
SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID  
NO:28, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ  
ID NO:34, SEQ ID NO:35, SEQ ID NO:148, SEQ ID NO:149, SEQ ID NO:150,  
SEQ ID NO:151, SEQ ID NO:153.

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6.- Microarray according to any of the claims 1 to 5 characterized by having the  
oligonucleotides coupled to a support.

7.- Use in extracorporeal methods of detection of mutations in LDL-r gene (SEQ  
25 ID NO: 1) for in vitro diagnosis of familial hypercholesterolemia of any of the  
oligonucleotides selected from: SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:16,  
SEQ ID NO:17, SEQ ID NO:24, SEQ ID NO:29, or at least one from SEQ ID  
NO:37 to SEQ ID NO:147 or from SEQ ID NO:154 to SEQ ID NO:259.

- 8.- Use in extracorporeal methods of detection of mutations in LDL-r gene (SEQ ID NO: 1) for in vitro diagnosis of familial hypercholesterolemia, according to claim 7 of any of the oligonucleotides selected from: SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:24, SEQ ID NO:29, or at least one from SEQ ID NO:37 to SEQ ID NO:147 or from SEQ ID NO:154 to SEQ ID NO:259, in combination with any of the oligonucleotides selected from: SEQ ID NO: 2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO: 9, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:148, SEQ ID NO:149, SEQ ID NO:150, SEQ ID NO:151, SEQ ID NO:153.
- 9.- Extracorporeal method of in vitro diagnosis of familial hypercholesterolemia characterized in that in a biological sample of an individual is detected in LDL-r gene (SEQ ID NO: 1), at least one mutation selected from: (-23)A>C, 1054 del11, 108delC, 1197de19, 1207de1T, 1432delG, 191-2delAinsCT, 2184delG, 231delC, 2399del5ins4, 313+insT, 338del16, 509insC, 675del15, 684dup12, 941-39>T, C195R, C255G, C319Y, D157G, D630N, E291X, H635N, N59K, T41M, W515X, Y379X, Y421X, T433N, 818de18, 1423delGC/insA, 1204insT, 451de13, G516X, 2389+4A>G, 1815del11, 1186+5G>A, T740M, 1771T, R279G, T446I, H562Q, C74Y, D686Y, G(-2)R, E579D, S205C, D200V, V766E, L(-6)P, 2544insC, C42Y, 2389+3A>C, [1587-5de15;1587del31].
- 10.- Extracorporeal method of in vitro diagnosis of familial hypercholesterolemia, according to claim 9, characterized in that in a biological sample of an individual, in combination with at least one of the mutations in LDL-r gene (SEQ ID NO: 1) selected from: (-23)A>C, 1054 del11, 108delC,

1197de19, 1207de1T, 1432delG, 191-2delAinsCT, 2184delG, 231delC, 2399del5ins4, 313+insT, 338dell6, 509insC, 675dell5, 684dup12, 941-39>T, C195R, C255G, C319Y, D157G, D630N, E291X, H635N, N59K, T41M, W515X, Y379X, Y421X, T433N, 818de18, 1423delGC/insA, 1204insT, 5 451de13, G516X, 2389+4A>G, 1815del11, 1186+5G>A, T740M, 1771T, R279G, T446I, H562Q, C74Y, D686Y, G(-2)R, E579D, S205C, D200V, V766E, L(-6)P, 2544insC, C42Y, 2389+3A>C, [1587-5de15;1587del31] is further detected, in the same LDL-r gene (SEQ ID NO: 1), at least one mutation selected from: 2393del9, (-42)C>G, (-49)C>T, 1045delC, 1061-8 T>C, A378T, C358R, 10 1358+1G>A, 1706-10G>A, 1845+1G>C, 2085del19, 211delG, 2140+5G>A, 2207insT, 2390-1G>C, 313+1G>C, 313+1G>A, 518delG, 7delC, 872delC, 884delT, 920ins4, A519T, C113W, C255X, C281Y, C297F, C347Y, C371X, C646Y, C677Y, C68W, C74G, C95R, D151N, D200G, D200Y, D280G, E10X, E246A, E256K, F634L, G322S, G352D, G571E, N543H, N804K, Q12X, 15 Q133X, Q357P, Q427X, Q71E, R395Q, R574W, R612C, S156L, S205P, T413K, T705I, V502M, W(-18)X, W541X, D679E, 1359-1G>A, C127R, 681ins21, C122X, V408M, G528D, D412H, N619N, E80K, L534P, L621S, C356Y, R329X, G248D, C201Y, 313+5G>A, C358Y, C331R, D157N, V776M, P664L, W462X, Q328X, L584P, R395W, G314V, W469X, P678L, R612H, R236W.

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11.- Extracorporeal method of in vitro diagnosis of familial hypercholesterolemia, according to any of the claims 9 or 10, characterized in that in a biological sample of an individual, in combination with at least one of the mutations in LDL-r gene (SEQ ID NO: 1) selected from: (-23)A>C, 1054 25 del11, 108delC, 1197de19, 1207de1T, 1432delG, 191-2delAinsCT, 2184delG, 231delC, 2399del5ins4, 313+insT, 338dell6, 509insC, 675dell5, 684dup12, 941-39>T, C195R, C255G, C319Y, D157G, D630N, E291X, H635N, N59K, T41M, W515X, Y379X, Y421X, T433N, 818de18, 1423delGC/insA, 1204insT, 451de13, G516X, 2389+4A>G, 1815del11, 1186+5G>A, T740M, 1771T, 30 R279G, T446I, H562Q, C74Y, D686Y, G(-2)R, E579D, S205C, D200V, V766E.



L(-6)P, 2544insC, C42Y, 2389+3A>C, [1587-5de15;1587del31], 2393del19, (-42)C>G, (-49)C>T, 1045delC, 1061-8 T>C, A378T, C358R, 1358+1G>A, 1706-10G>A, 1845+1G>C, 2085del19, 211delG, 2140+5G>A, 2207insT, 2390-1G>C, 313+1G>C, 313+1G>A, 518delG, 7delC, 872delC, 884delT, 920ins4, A519T, C113W, C255X, C281Y, C297F, C347Y, C371X, C646Y, C677Y, C68W, C74G, C95R, D151N, D200G, D200Y, D280G, E10X, E246A, E256K, F634L, G322S, G352D, G571E, N543H, N804K, Q12X, Q133X, Q357P, Q427X, Q71E, R395Q, R574W, R612C, S156L, S205P, T413K, T7051, V502M, W(-18)X, W541X, D679E, 1359-1G>A, C127R, 681ins21, C122X, V408M, G528D, D412H, N619N, E80K, L534P, L621S, C356Y, R329X, G248D, C201Y, 313+5G>A, C358Y, C331R, D157N, V776M, P664L, W462X, Q328X, L584P, R395W, G314V, W469X, P678L, R612H, R236W, is further detected at least one LDL-r gene (SEQ ID NO: 1) polymorphism selected from: 81T>C BstUI Exon 2, 1060+10G>C SmaI Exon 7, 1171G>A StuI Exon 8, 1413G>A DdeI Exon 10, 1617C>T BstNI Exon 11, 1725C>T SSCP Exon 12, 1771C>T HincII Exon 12, 1959 T>C AvaII Exon 13, 2232G>A MspI Exon 15.

12.- Extracorporeal method of in vitro diagnosis of familial hypercholesterolemia according to any of the claims 9 to 11, comprising amplifying DNA fragments that contain any mutation in LDL-r gene (SEQ ID NO: 1) selected from: (-23)A>C, 1054 del11, 108delC, 1197de19, 1207de1T, 1432delG, 191-2delAinsCT, 2184delG, 231delC, 2399del5ins4, 313+1insT, 338del16, 509insC, 675del15, 684dup12, 941-39>T, C195R, C255G, C319Y, D157G, D630N, E291X, H635N, N59K, T41M, W515X, Y379X, Y421X, T433N, 818de18, 1423delGC/insA, 1204insT, 451de13, G516X, 2389+4A>G, 1815del11, 1186+5G>A, T740M, I771T, R279G, T446I, H562Q, C74Y, D686Y, G(-2)R, E579D, S205C, D200V, V766E, L(-6)P, 2544insC, C42Y, 2389+3A>C, [1587-5de15;1587del31], alone or in combination with any mutation in LDL-r gene (SEQ ID NO: 1) selected from: 2393del19, (-42)C>G, (-49)C>T, 1045delC, 1061-8 T>C, A378T, C358R, 1358+1G>A, 1706-10G>A, 1845+1G>C, 2085del19.

211delG, 2140+5G>A, 2207insT, 2390-1G>C, 313+1G>C, 313+1G>A, 518delG, 7delC, 872delC, 884delT, 920ins4, A519T, C113W, C255X, C281Y, C297F, C347Y, C371X, C646Y, C677Y, C68W, C74G, C95R, D151N, D200G, D200Y, D280G, E10X, E246A, E256K, F634L, G322S, G352D, G571E, N543H, N804K, Q12X, Q133X, Q357P, Q427X, Q71E, R395Q, R574W, R612C, S156L, S205P, T413K, T7051, V502M, W(-18)X, W541X, D679E, 1359-1G>A, C127R, 681ins21, C122X, V408M, G528D, D412H, N619N, E80K, L534P, L621S, C356Y, R329X, G248D, C201Y, 313+5G>A, C358Y, C331R, D157N, V776M, P664L, W462X, Q328X, L584P, R395W, G314V, W469X, P678L, R612H, R236W and/or any polymorphism in LDL-r gene (SEQ ID NO: 1) selected from: 81T>C BstUI Exon 2, 1060+10G>C SmaI Exon 7, 1171G>A StuI Exon 8, 1413G>A Ddel Exon 10, 1617C>T BstNI Exon 11, 1725C>T SSCP Exon 12, 1771C>T HincII Exon 12, 1959 T>C AvaII Exon 13, 2232G>A MspI Exon 15, by the technique of the chain reaction of the polymerase (PCR), utilizing therefore any of the oligonucleotides selected among SEQ ID NO:2 to SEQ ID NO:259 or combinations of the same, subjecting the PCR products to an analysis by the simple chain conformation polymorphisms technique (SSCP), sequencing those fragments having an anomalous pattern by SSCP to detect the mutations, that would be identified subsequently by restriction analysis or by means of the microarray of claims 1 to 6.

13.- Oligonucleotides able of specifically detecting in LDL-r gene (SEQ ID NO: 1) any of the mutations selected from: (-23)A>C, 1054 del11, 108delC, 1197de19, 1207de1T, 1432delG, 191-2delAinsCT, 2184delG, 231delC, 2399del5ins4, 313+1insT, 338dell6, 509insC, 675dell5, 684dup12, 941-39>T, C195R, C255G, C319Y, D157G, D630N, E291X, H635N, N59K, T41M, W515X, Y379X, Y421X, T433N, 818de18, 1423delGC/insA, 1204insT, 451de13, G516X, 2389+4A>G, 1815del11, 1186+5G>A, T740M, 1771T, R279G, T446I, H562Q, C74Y, D686Y, G(-2)R, E579D, S205C, D200V, V766E, L(-6)P, 2544insC, C42Y, 2389+3A>C, [1587-5de15;1587del31].

14.- Oligonucleotides according to claim 13 selected from: SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:24, SEQ ID NO:29, or at least one from SEQ ID NO:37 to SEQ ID NO:147 or from SEQ ID NO:154 to  
5 SEQ ID NO:259.